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Liver Initiation Assay: The Rat Liver Foci Bioassay of Carcinogens and Noncarcinogens

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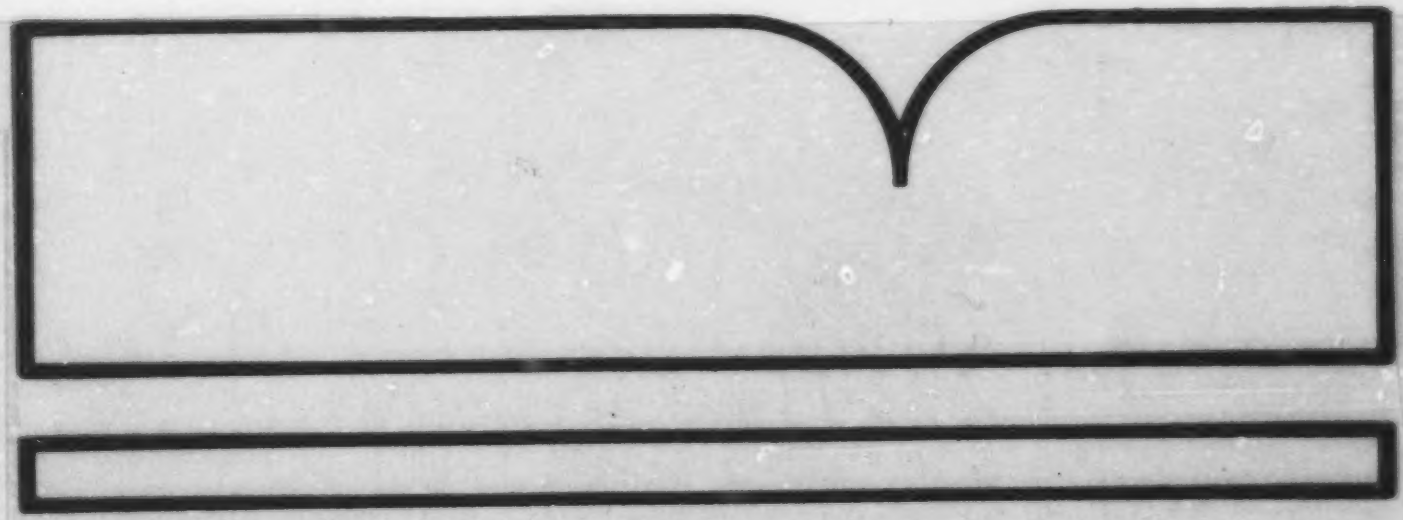

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LIVER INITIATION ASSAY: THE RAT LIVER FOCI BIOASSAY OF CARCINOGENS AND NONCARCINOGENS

by

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16. ABSTRACT

2-Acetylaminofluorene, 4-acetylaminofluorene, benzo(a)pyrene and pyrene were evaluated in the rat liver foci bioassay for their ability to initiate gamma-glutamyltranspeptidase-positive foci. Under the conditions used, 2-acetylaminofluorene and benzo(a)pyrene were active in the assay while 4-acetylaminofluorene and pyrene were not active.

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SUMMARY:

2-Acetylaminofluorene, 4-acetylaminofluorene, benzo(a)pyrene and pyrene were evaluated in the rat liver foci bioassay for their ability to initiate gamma-glutamyltranspeptidase-positive foci. Under the conditions used, 2-acetylaminofluorene and benzo(a)pyrene were active in the assay while 4-acetylaminofluorene and pyrene were not active.

This report contains the results of our participation in the International Collaborative Study to evaluate why some genotoxic chemicals were inactive in *in vivo* rodent carcinogenesis bioassays. 4-Acetylaminofluorene and pyrene, two genotoxic chemicals that have been inactive in *in vivo* rodent carcinogenesis bioassays, and their genotoxic and carcinogenic analogues, 2-acetylaminofluroene and benzo(a)pyrene, were evaluated in the rat liver foci bioassay for tumor initiating activity. The rat liver foci assay has been proposed for use to demonstrate the carcinogenic potential of chemicals, especially those determined to be genotoxic (Bull and Pereira, 1982; Weisburger and Williams, 1983). The substance under test is administered to rats that have received a partial hepatectomy so that the first peak of regenerative DNA synthesis occurs during the period of maximum binding of the test substance to cellular DNA. Thus, an attempt is made to maximize the extent of fixation in the daughter strands of the alteration crucial for the initiation of carcinogenesis. The fact that the time of maximum DNA binding for most test substance is not known has resulted in the need to evaluate the activity of a test substance by administering it at different times after the partial hepatectomy in this bioassay. After a period of 1-2 weeks for the liver to recuperate from the toxicity of the test substance, the animals received 500 ppm sodium phenobarbital in their drinking water in order to stimulate the initiated cells to grow into a clone of altered hepatocytes. These clones or foci of initiated cells can be recognized by alteration in their phenotype such as an increase in gamma glutamyltranspeptidase (GGT) activity (Pugh and Goldfarb, 1978; Pitot and Sirica, 1980), a decrease in adenosine triphosphate (Scherer and Emmelot, 1976), a decrease in glucose-6-phosphatase activity (Tatematsu *et al*. 1977) or a decrease in their ability to accumulate iron (Williams, 1980). It is believed that the presence of

these altered-foci demonstrate the occurrence of initiation of carcinogenesis and can predict the eventual appearance of tumors (Farber, 1980; Pitot and Sirica, 1980; Pereira, 1984). Therefore, the ability of a substance to initiate the formation of altered-foci has become the basis of a short-term *in vivo* bioassay in rat liver for the determination of whether a genotoxic chemical is capable of initiating carcinogenesis.

MATERIAL AND METHODS

Animals

Male Fisher-344 rats were purchased from Charles River Co. (Portage, MI). The animals received rodent laboratory chow (Ralston Purina Co., St. Louis, MO) and drinking water *ad libitum*.

Materials

Diethylnitrosamine (DENA) was purchased from Eastman Kodak Co. (Rochester, NY); N-gamma-L-glutamyl-4-methoxy-2-naphthylamide from Bachem (Torrence, CA); and sodium phenobarbital from J. T. Baker (Philipsburg, NJ). 2-Acetylaminofluorene (2-AAF), 4-acetylaminofluorene (4-AAF), benzo(a)pyrene (BaP) and pyrene were supplied by the International Programme on Chemical Safety/ World Health Organization.

Experimental Design

The protocol of the rat liver foci bioassay is presented in Figure 1. The rats, weighing 200-225 gm at the start of the study, received while under ether anesthesia either a 2/3 partial hepatectomy or a sham operation. The sham operation consisted of externalizing and then returning the liver to the peritoneal cavity. The test chemical was dissolved in corn oil on the day of treatment and administered orally by stomach tube either 6 or 12 hours after the operation. Each animal received 2 ml/kg corn oil. Some animals received diethylnitrosamine which is used in our laboratory as a positive control

to ensure that the sensitivity of the assay has not changed. Seven days after the operation, the animals started to receive 500 ppm sodium phenobarbital in their drinking water for the next 70 days. Seventy-seven days after the operation, the sodium phenobarital was removed and the animals killed 7 days later by decapitation. Four tissue blocks, 2 each from the remaining 2 lobes were taken and quickly frozen on dry ice in Optimum Cutting Temperature Compound (Fisher Chemical Co. Pittsburgh, Pa). Sections, 8 μ thick were obtained and stained for GGT activity by the procedure of Rutenburg et al. (1969) and counterstained with hemotoxylin. Figure 2 depicts a liver section containing GGT-foci.

Three sections totaling 2-4 cm^2 were evaluated from each animal for the presence of GGT-foci. The area of each focus and section were obtained by projection of the image onto a digitizing broad (Elographics, Oak Ridge, TN), connected to a WANG Model 2200 VP computer (Lowell, MA). The number of foci/cm^3 was calculated as described by Campbell et al. (1982).

RESULTS

The results of the rat liver foci bioassay are presented in Table 1. 2-Acetylaminofluroene, benzo(a)pyrene and diethylnitrosmaine were active in the bioassay, while 4-acetylaminofluorene (1.35 mmole/kg) and pyrene (1.50 mmole/kg) were inactive. The minimum tested dose of 2-AAF (0.045 mmole/kg), B(a)P (0.08 mmole/kg) and DENA (0.03 m mole/kg) were positive in the assay so that the assay should be capable of detecting even lower doses of these chemicals.

The rat liver foci assay was more sensitive to B(a)P administered 12 rather than 6 hours after a partial hepatectomy. No consistent difference in the sensitivity of the assay was observed between 2-AAF administered at either 6 or 12 hours after a partial hepatectomy or between diethylnitrosamine administered at either 12 or 18 hours. 2-AAF and B(a)P were not active

in the assay when administered to sham operated animals while diethylnitrosamine was active in sham operated animals, although at a lower level of activity.

DISCUSSION

Initiation of chemical carcinogenesis requires the simultaneous occurrence of DNA alteration/binding and DNA replication. The liver has a very low level of DNA replication and cellular proliferation so that initiation in the liver requires the induction of DNA replication. The induction of DNA replication can result from the mitogenic action of a chemical or from the regenerative response to the toxicity of a chemical. In the rat liver foci bioassay, a 2/3 partial hepatectomy is performed and the resulting regenerative growth of the liver is used to fix the alteration in the DNA into the daughter strands. Thus, initiation in the rat liver foci bioassay requires that the test substance binds/alters DNA in the crucial manner for initiation to occur while no longer requiring that it stimulate DNA replication because of the regenerative response to the partial hepatectomy. This results in the rat liver foci bioassay being sensitive to chemicals such as B(a)P, which in intact animals are not carcinogenic toward the liver (Craddock, 1976; Tsuda et al., 1980; Herren et al., 1982).

The apparent requirement for a substance to be active in the rat liver foci bioassay is that it binds DNA in such a manner as to be genotoxic and thereby initiate carcinogenesis. In the International Collaborative Program to Evaluate Short-Term Tests for Carcinogens, 4-AAF and pyrene demonstrated genotoxic activity in bacterial mutation and eukaryotic systems (Ashby, 1981). Hence, it appears that these 2 chemicals, which were not active in prior long-term rodent carcinogenesis bioassays, are capable of producing genotoxic alterations in DNA. 4-AAF was less than 1/30 as active as 2-AAF in

the rat liver foci bioassay since 1.35 mmole/kg 4-AAF was inactive while 0.045 mmole/kg 2-AAF was active in the assay. Pyrene was less than 1/20 as active as B(a)P in the assays.

The inactivity of 4-AAF and pyrene in our study could have resulted either from them being noncarcinogens or from them possessing less activity than their carcinogenic analogues. If the latter case is true, they may not have been tested in our study at a dose sufficient to demonstrate their activity in the rat liver foci bioassay. In the bacterial mutation assays for carcinogens, 2-AAF was approximately 30-100 times more active than 4-AAF (Bridges et al., 1981). In the rat liver foci bioassay, 4-AAF was not tested at a dose which was greater than 30-100 times the 2-AAF dose. As a result, if 4-AAF has the same relative activity as 2-AAF in the rat liver foci bioassay as in the bacterial mutation assays, then 4-AAF would have been tested in the liver foci bioassay at doses below the sensitivity of the assay. Thus, we are unable to conclude that 4-AAF as well as pyrene, are noncarcinogens instead of weak carcinogens that possess activity below the sensitivity of the bioassay as performed in our study.

The potency of a chemical in the rat liver foci bioassay is dependent on the extent of crucial alterations produced in the DNA. Thus, it is expected that a relationship (possibly linear) should exist between the response in the rat liver foci bioassay and the amount of genotoxic adduct formed in DNA which is crucial for initiation of carcinogenesis. It is recognized that the rate of repair of the genotoxic adduct and possibly other pharmacokinetic parameters would alter this relationship. However, it would be very productive to compare in rat liver the extent of DNA adduct formation for 4-AAF and 2-AAF and for pyrene and B(a)P. If equal potency in the rat liver foci bioassay is assumed for the adducts of the noncarcinogenic and of the carcino-

genic analogue, one could estimate whether the extent of DNA alteration induced by the noncarcinogenic analogue was sufficient for activity in the assay. If sufficient DNA binding occurs with the noncarcinogenic analogue, then another property would have to be lacking thereby resulting in its inability to initiate carcinogenesis even though it is genotoxic.

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TABLE 1

RAT LIVER FOCI BIOASSAY

Chemical[a] mmole/kg	GGT-Foci/cm^3			
	Partial Hepatectomized		Sham Operation	
	6 hrs.	12 hrs.	6 hrs.	12 hrs.
2-Acetylaminofluorene				
0.045	679 ± 221 (6)[b]	370 ± 90.5 (9)	N.D.	N.D.
0.225	698 ± 210 (6)	1208 ± 246 (6)	N.D.	N.D.
0.450	1030 ± 249 (7)	588 ± 196 (9)	N.D.	N.D.
1.35	424 ± 233 (4)	1013 ± 288 (9)	80.6 ± 54.4 (10)	74.0 ± 74.0 (9)
4-Acetylaminofluorene				
0.225	38.2 ± 29.3 (9)	20.9 ± 20.9 (8)	N.D.	N.D.
1.35	21.7 ± 21.7 (4)	110 ± 110 (4)	0 ± 0 (10)	105 ± 75.6 (7)
Benzo(a)pyrene				
0.08	39.8 ± 39.8 (6)	196 ± 125 (4)	N.D.	N.D.
0.40	254 ± 120 (7)	513 ± 322 (5)	N.D.	N.D.
1.20	341 ± 134 (6)	538 ± 105 (5)	0 ± 0 (8)	22.8 ± 22.8 (6)

(Table 1 - Continued)

Pyrene				
0.50	0 ± 0 (7)	90.4 ± 25.4 (5)	N.D.	N.D.
1.50	0 ± 0 (8)	71.0 ± 71.0 (5)	0 ± 0 (8)	29.6 ± 20.8 (8)
Corn Oil				
2 ml/kg	189 ± 130 (5)	0 ± 0 (7)	78.5 ± 61.8 (6)	0 ± 0
Diethylnitrosamine	**12 hrs.**	**18 hrs.**	**12 hrs.**	**18 hrs.**
0.030	1470 ± 448 (6)	1120 ± 287 (6)	N.D.	N.D.
0.060	1280 ± 646 (6)	1800 ± 348 (4)	751 ± 413 (6)	491 ± 285 (4)

a. The chemicals were administered either 6 or 12 hours after a 2/3 partial hepatectomy or a sham operation except for diethylnitrosamine which was administered either 12 or 18 hours after the operation.

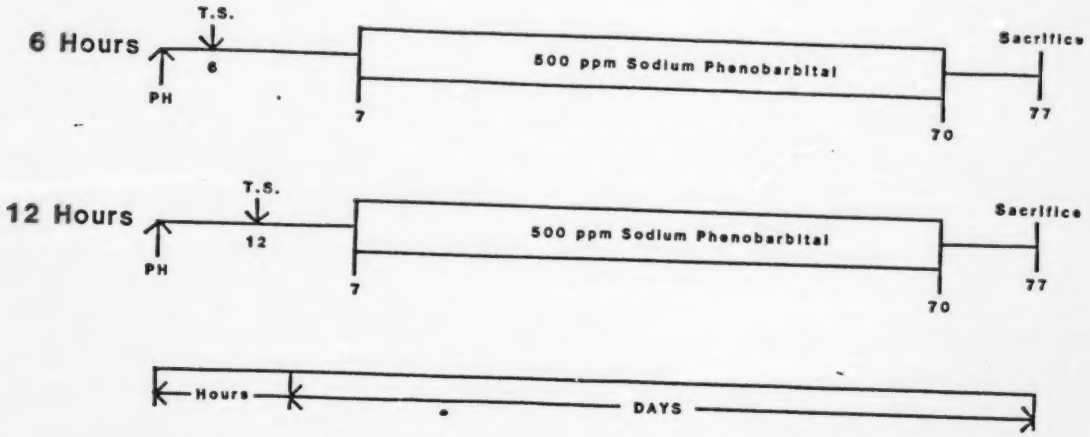
b. Results are means ± S.E. for the number of animals in parenthesis.

c. N.D., not determined.

FIGURES

Figure 1. Experimental protocols for the rat liver foci bioassay in which the chemical under test were administered either 6 or 12 hours after a two-thirds partial hepatectomy.

EXPERIMENTAL PROTOCOLS



PH - Partial Hepatectomy

T.S. - Test Substance